United States Environmental Protection Agency

LABORATORY ANIMAL PROJECT REVIEW

Please note:

1. All information in this LAPR is considered privileged and confidential by the IACUC and regulatory authorities.
2. Approved LAPRs are subject to release to the public under the Freedom of Information Act (FOIA). Do not include proprietary or classified information in the LAPR.
3. An approved LAPR is valid for three years.

LAPR Information

LAPR Title:	Mouse Strain Comparison of Airway Responses During and After Repeated Ozone Exposure Using Three Methods of Pulmonary Function Assessment
LAPR Number:	18-09-004
Principal Investigator	Exemption 6
Author of this Document:	Exemption 6/RTP/USEPA/US
Date Originated:	09/02/2015
LAPR Expiration Date:	09/30/2018
Agenda Date:	09/09/2015
Date Approved:	09/28/2015
Date Closed:	

APPROVALS

APPROVER	NAME	APPROVAL DATE	COMMENTS	
	Exemption 6/RTP/USEPA/US by Exemption 6/RTP/USEPA/US	09/28/2015	DMR	
	Exemption 6 Exemption 6 Exemption 6 RTP/USEPA/US by Exemption 6/RTP/USEPA/US	09/28/2015	DMR	

Administrative Information

1. Project Title (no abbreviations, include species):
Mouse Strain Comparison of Airway Responses During and After Repeated Ozone Exposure Using Three Methods of Pulmonary Function Assessment

Is this a continuing study with a previously approved LAPR?
No

2. Programatic Information

a. What Program does this LAPR support? Please provide the Research Program, Project, Task Number and Title.
This research is categorized under the NHEERL Air, Climate and Energy (ACE) research program, PEP-1 project, a New ACE Task on "Single and Co-pollutant Effects of HAPs and Criteria Pollutants as Determinants of Health Outcomes in Near-Source Environments".

b. What is the Quality Assurance Project Plan (QAPP) covering this project?
The IRP encompassing this research is - IRP-NHEERL-RTP/EPHD/CIB[redacted]/2014-001-r1.

3. EPA Principal Investigator/Responsible Employee:

Principal Investigator	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	Exemption 6	EPHD	MD
	Lotus Notes Address	***Branch***	
	Exemption 6 Exemption 6 Exemption 6 Exemption 6 RTP/USEPA/US	CIB	

4. Alternate Contact:

Alternate Contact	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	Exemption 6	EPHD	MD
	Lotus Notes Address	***Branch***	
	Exemption 6 Exemption 6 Exemption 6/RTP/USEPA/US	CRB	

SECTION A - Description of Project

1. Explain the study objective(s) in <u>non-technical language</u> such that it is understandable by non-scientific persons. <u>Explain how the benefits from the knowledge gained from this research outweigh the costs to the animals used in this research.</u> If this is a continuing study from a previous LAPR, briefly justify the continuation. Please spell out all acronyms and abbreviations with their initial use.

Both asthma and obesity rates have increased significantly in children over the past several decades. As such, the increases in asthma and obesity are likely to be connected in a multifactorial manner, with co-morbidities related to lifestyle (poor diet, lack of physical activity) and early life insults (infections and air pollution) contributing in some capacity. It is not at all clear as to whether obesity directly leads to increased airway reactivity, or whether obesity serves to promote airway inflammation, with secondary changes in airway responsiveness. In order to better assess the role of air pollution in the development of asthma and obesity, appropriate murine models are needed. Moreover, it will be necessary to accurately, non-invasively and repeatedly assess lung functional changes and airway responsiveness as the same animal undergoes repeated or subchronic air pollutant exposure.

To date, we have relied on whole body plethysmography (WBP) to non-invasively estimate changes in ventilation (breathing frequency and breath volumes). While WBP assessments are quite straightforward in health, during bronchoconstriction or during reduced ventilation (common in mice exposed to irritants), their utility is less predictable. Therefore, researchers at EPA have invested in a limited number of double-chamber plethysmographs as this approach reportedly provides more accurate ventilatory measurements as well as airway resistance (sRaw) estimates.

In this initial project, we will directly compare lung ventilatory changes in mice exposed to a known irritant pollutant, ozone, as measured using whole body plethysmography (WBP) vs. double chamber plethysmography (DCP). We will compare two strains of mice that are commonly used in air pollution studies, the C57BL/6 and the BALB/c strains. Results obtained using non-invasive WBP or DCP after a single 2 hr ozone (0.8ppm) exposure or after repeated ozone exposure (once per week x 4 weeks) will be compared to the “gold standard” but non-survival flexivent pulmonary function testing system.

Classic whole body plethysmography assesses breathing parameters which are a combination of airflow in the head and body, which nearly cancel each other out but still provide a muted signal. Double chamber plethysmography separates the signal of the head from that of the thoracic wall movement and thus provides a much stronger signal as well as provides calculated parameters such as specific airway resistance. This procedure should be much more sensitive for detecting small changes in pulmonary function occurring for example, as a result of air pollutant exposure. By comparison, the Flexivent method of assessing pulmonary function is based on forced oscillometry testing (generation of sound waves and assessing their "return" to quantify airway patency) (http://www.jove.com/video/50172/evaluation-respiratory-system-mechanics-mice-using-forced-oscillation). As such it provides the mst objective or quantitative measures of pulmonary pressure changes and dynamic compliance changes. However, in order to perform forced oscillometry maneuvers, a cannula must be securely placed directly into the trachea (via a surgical procedure referred to as a tracheostomy). In addition, the subject is paralyzed and ventilated through the tracheal cannula so that spontaneous breathing efforts do not interfere with the forced oscillometry maneuvers. Animals are not recovered from this procedure so it is not possible to perform lung function testing with this approach more than once during a study.

2. Scientific rationale for proposed animal use.

a. Why is the use of animals necessary?

The use of animals is necessary in order to understand the complex physio-logi-cal responses occurring during and after exposure to air pollution, in particular changes in lung function and airway responsiveness in healthy animals or in animal models of metabolic or allergic airways disease. Validated in vitro methods for assessing pollutant-induced lung functional changes have not been conclusively demonstrated. Following a bibliographic search in PubMed, no validated accepted non-animal methods have been identified to properly mimic inhalation exposures and the subse-quent development of complex metabolic, immune, and pulmonary function changes that may culminate in and contribute to chronic lung disease in humans.

b. Justify the species requested:

The toxicological literature shows how mice exposed acutely to photo-chemical pollutants develop lung injury, inflammation and related pathophysiological endpoints, thus resembling changes occurring after acute exposure of

humans to like pollutants. Numerous murine strains/models exist and have been incorporated in these approaches. Herein, we propose to focus on two inbred strains, the C57BL/6 and the BALB/c.

The C57BL/6 strain remains one of the most commonly used strains in part related to serving as the "background" strain for a variety of transgenic mice. The C57BL/6 stain is also notable for its utility in investigating obesity-related metabolic disorders and Th1-immune dysregulation.

One the other hand, the BALB/c strain is notable for its use regarding Th2-type cytokine polarization (i.e., an immunologic shift to a pro-allergic state), and is therefore commonly used as a model for allergic airway disease(s). Like asthmatic humans, sensitized BALB/c mice develop eosinophilic airway inflammation (eosinophils are white blood cells which release a variety of mediators which promote airway constriction), lung edema (fluid in the airways), elevated serum IgE (the primary allergic antibodies which promote severe allergic reactions), increased allergic cytokines (especially IL-5, a molecular mediator of inflammation), and increased airway responsiveness to non-specific agonists (i.e., methacholine which induces narrowing of the airways). Importantly, well defined, commercially available immune and molecular reagents as well as many standard operating procedures are available for the mice in these studies.

3. How was it determined that this study is not unnecessary duplication?

While Pubmed and literature searches in Google and PubMed performed in August and September 2015 identify several studies assessing "ozone" effects in mice, direct comparisons of lung functional changes across all 3 tests systems are limited and somewhat in conflict. No reports on mouse strain compari-sons (C57BL/6 and the BALB/c) with DCP during and after ozone exposure were identified.

SECTION B - In Vivo Procedures

1. Briefly describe the experimental design. Include descriptions of the age, weight and sex of the animals. Supplementary information may be attached at the end of the LAPR, but please include critical information within the body of the LAPR.

Mice – female, young adult, 6- 8 week old. Approx 18 - 20 gm.

Experimental objectives of this study are:

Objective 1: Obtain and compare real-time ventilatory measurements during and after pollutant exposures across 2 mouse strains using WBP and DCP during acute (single) ozone exposure or repeated ozone exposure (once/week for 4 weeks).

Objective 2: Validate the double-chamber assessment vs. the forced oscillometry-derived "gold standard" pulmonary function ("Flexi-vent") assessment.

Objective 3: Assess strain differences in airway ventilation and responsiveness that may be relevant for development of future obesity models (e.g., high caloric/fat diets), allergic lung disease models (e.g., sensitization to respiratory allergens), or combined morbidities.

Objective 4: Improve bronchoalveolar lavage recovery post-bronchoconstriction (i.e., after methacholine challenge) by administering an anticholingeric agent prior to necropsy and lung lavage.

2. Justify the number of animals. Include explanation (e.g., biological, statistical, regulatory rationale) for the number of animals needed for each treatment group, and the overall number requested for the duration of the LAPR.

For WBP, to assure that we reach the appropriate numbers to achieve statistical significance, using the C57BL/6 mice, this study will require n=10/air and n=10/ ozone group for the single exposure subset (20 total) plus n=10/air and n=10/ozone for the repeated 4 week exposure subset (another 20 total).
Hence, 20 + 20 = 40 C57BL/6 mice for WBP testing.

For the DCP assessment, using the C57BL/6 mice, this study will require n=10/air and n=10/ozone group for

the single exposure subset (20 total) plus n=10/air and n=10/ozone group for the repeated 4 week exposure subset) (another 20 total). 20 + 20 = 40 C57BL/6 mice for DCP testing.
In total, 40 + 40 = 80 C57BL/6 mice.

For WBP testing of BALB/c mice, the study will require n = 40 mice.

For DCP testing of BALB/c mice, the study will require n = 40 mice.
In total, 40 + 40 = 80 BALB/c mice.

3. State how many animals over the study period are expected to be used under the following three categories of pain/distress (USDA nomenclature as defined in the instructions): Please enter numbers only.

Categories	Adults	Offspring
C) Minimal, transient, or no pain/distress:		
D) Potential pain/distress relieved by appropriate measures:	160	
E) Unrelieved pain/distress:		

4. Does this LAPR include any of the following:

☐ Restraint (>15 Minutes) ☐ Survival surgery
☐ Food and/or water restriction (>6 Hours) ☒ Non-survival surgery

a. Please provide a scientific justification. Describe how animals will be monitored, how health status will be tracked, and what records will be maintained.

Non-survival flexivent pulmonary function testing maneuvers in mice is added here because this is the only protocol that can provide objective measures of pulmonary pressure and dynamic compliance which leads to calculation of airway resistance. Airway responsiveness to Methacholine (MCh) aerosol will be assessed with the Flexivent system. To do so, mice will anesthetized (one at a time) with urethane (1 g/kg intraperitoneal, about 0.3 ml for a 20 gm mouse) and carefully tested to determine lack of responses to toe pinch. (Urethane is known to preserve cardiovascular function better than pentobarbital).

To perform Flexivent testing, a secure tracheal cannula must be place. The tracheotomized animal is then connected to the ventilator via the canula luer, on a 37 degree C heated pad (the pad is heated by circulating water kept at 37 C). Mice are ventilated with 100% oxygen at 150 breaths per minute. The tail is taped to the heating pad to ensure the animal is kept in a straight line with the ventilator, but the 4 limbs are not restrained. Electrical leads are attached to three of the animal's limbs, which allow heart rate and waveform to be monitored by a window on the computer monitor. A rectal probe is then inserted to monitor body temperature. Monitoring the temperature, heart rate and waveform, along with the characteristics of the airway pressure tracing in a separate window, allows us to be sure that the animal is in a sufficient state of anesthesia after administration of the pancuronium bromide. Each mouse is then paralyzed with pancuronium bromide (0.8 mg/kg i.p., about 0.2 ml for a 20 gm mouse) to eliminate skeletal muscle movement which interferes with airway measurements.

Animals will be monitored during the procedure and should they show any signs of recovery from anesthesia, (e.g. eye and body movement) then the animal will be euthanized by overdose of sodium pentobarbital (> 200 mg/kg, i.p.).

Data will be stored in corresponding lab notebooks or electronic data files.

5. Category C procedures. Describe each procedure separately, include details on the following:

a. Treatments (e.g., dosages, duration of exposure, route, volume, frequency):

General ozone inhalation exposure conditions:

Subsets of C57BL/6 and BALB/c mice will undergo a single air or ozone (0.8 ppm) inhalation exposure for 2 hours/day – either in whole-body plethysmographs or in double-chamber plethysmographs.

Other subsets of C57BL/6 and BALB/c mice will undergo repeated air or ozone (0.8 ppm) inhalation exposure for 2 hours/once per week for 4 consecutive weeks) – either in whole-body plethysmographs or in double-chamber plethysmographs.

Food and water are withheld while mice are being exposed. Mice will be weighed after each ozone exposure, and examined for any visible clinical signs of discomfort or poor health. All findings are recorded. Cumulatively, mice will receive a total of 8hr of 0.8 ppm ozone exposure over a 4 week period.

Air-exposed control mice will be exposed to filtered air under the same conditions alongside ozone exposed mice.

b. Survival Blood Collections (method, volume, frequency):
none
c. Testing methods (including non-stressful dietary restrictions/modifications, mild non-damaging electric shock):
Whole Body

For measurement of breathing parameters in whole body plethysmographs, animals will be placed in individual WBP (formerly referred to "BUXCO") chambers where they have freedom of movement in a chamber 3 inches diameter by 2 inches height. We may monitor breathing in the chamber for up to 25 minutes immediately before and after exposures. Monitoring of breathing parameters may be continued up to 1 day post-exposure (no more than 2 other times post-exposure after the immediate response; each time for only 25 minutes. This is a well-tolerated procedure which we have conducted for many years and we have not noted any problems. No drug treatment (e.g. methacholine aerosol) will be administered during this monitoring).

Double Chamber:

EPA researchers have acquired 4 double chamber mouse plethysmographs from emka Technologies, which are vastly improved in design over the traditional double-chamber plethysmograph. In older versions of double chamber plethysmographs, a lab animal is placed in a plethysmograph separated into head and body chambers by a circular cutout wall and made airtight with a latex seal around the neck. The stress of the seal around the neck is evident and the animal may struggle, turn around, or chew on the seal, often causing unreliable measurements. The new design simply restrains the mouse in a conical tube very similar to a nose-only exposure tube. No neck seal is needed, and flow transducers placed at the body chamber and head chamber allow separate measures of airflow for the head and thorax and reliable airflow measurements including specific airway resistance. This procedure has been previously approved for use in mice under a separate LAPR (Exemption 6 17-04-002). As indicated in that LAPR, herein we will acclimate the mice to adapt to the tubes, similar to what is done for nose-only exposures. The acclimation period will include placement into tubes for increasing time periods as follows: 5 minutes/session (Day 1 am), then 15 minutes/session (Day 1 pm), 30 minutes/session (Day 2 am), then 30 minutes/session (Day 2 pm), for a total of 4 acclimation sessions.

On Day 3 (am), mice will undergo air or ozone-exposure while within the WPB or DCP for 2 hr. As the DCP method is nearly identical to nose-only exposure (and the restraint is for a limited time compared to typical nose-only exposures), this procedure is categorized as Category C. We will however carefully monitor the mice during the testing of the system. If the animal shows signs of significant stress (excessive struggling or excretion), we will halt the testing and assess the animal. Based on many years of experience with nose-only exposures we do not expect significant effects. To recap, the primary purpose of this study is simply to test airway and ventilatory responses obtained via the double chamber plethysmographs to responses obtained using whole body plethysmography.

d. Animal restraint and confinement beyond routine housing and handling. Include a description of the type of restraint device, acclimation to device, duration of restraint:
See description of acclimation and testing while in the double chamber plethysmograph (above, section c).
e. Breeding for experimental purposes (e.g. length of pairing, number of generations):
None.
f. Describe how animals will be identified and monitored. Include description of identification procedures. (For example, if transponders are used, how are the animals prepared?) Include frequency of observations and by whom:
Mice may be tagged with ear buttons or other identification options. During exposure Exemption 6 Exemption 6 Exemption 6 will monitor animals, at least once per half-hour for entire 2h exposure duration. During non-exposure periods, mice will be monitored once daily for visible signs of discomfort. Body weight will be

obtained at least weekly. No weight loss or significant distress is expected with any of the experimental conditions.

6. Non-surgical Category D or E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9).

a. Treatments (e.g. dosages, duration of exposure, route, volume, frequency):
none

b. Blood Collection (Provide a description of the procedure including method, volume, and frequency if appropriate. Indicate if the procedure is survival or terminal. Include preparatory methods, descriptions of incisions, etc.):
n/a

c. Testing methods:
n/a

d. Restrictions placed on the animals' basic needs (e.g., food and/or water restriction, light cycles, temperature). Provide details regarding the length of restriction. Describe the method(s) for assessing the health and well-being of the animals during restriction. (Amount of food or fluid earned during testing and amount freely given must be recorded and assessed to assure proper nutrition.):
n/a

e. Describe how animals will be monitored (e.g., frequency of observations, by whom):
n/a

f. Analgesia (Category D Procedures) - list drugs, dosages, route of administration and frequency:
n/a

g. If treatment-related deaths are expected, this must be thoroughly justified. Death as an endpoint is highly discouraged:
n/a

7. Surgical Category D and E procedures. Indicate if the surgery is survival or terminal. Describe each surgical procedure separately, include details on the following (Also fill in Section B.9)

a. Complete description of surgical procedure including presurgical preparation, aseptic technique, surgical closure, etc:
After the final air or ozone exposure, the lung functional measurements obtained in the whole-body (WBP) or double chamber plethysmographs (DCP) will be validated by assessing these same mice in the "flexi-vent" system (the "gold" standard forced oscillometry-derived pulmonary function assessment). Mice will either be evaluated using the single or multi-chamber (4) system as available (see descriptions below).

Single mouse:

Non-survival flexivent pulmonary function testing maneuvers in mice is added here because this is the only protocol that can provide objective measures of pulmonary pressure and dynamic compliance which leads to calculation of airway resistance. Airway responsiveness to Methacholine (MCh) aerosol will be assessed with the Flexivent system. To do so, mice will anesthetized (one at a time) with urethane (1 g/kg intraperitoneal, about 0.3 ml for a 20 gm mouse) and carefully tested to determine lack of responses to toe pinch. (Urethane is known to preserve cardiovascular function better than pentobarbital). This procedure takes approximately 30 minutes to complete, from initial anesthesia to the recording of the response following the last exposure to methacholine aerosol.

The instruments and surrounding area will be clean and the surgeon (**Exemption 6**) will wear gloves. The tracheotomy procedure is as follows: The fur is clipped in the neck area with a small electric clipper, and then 70% alcohol is applied to the area. Small surgical scissors are used to cut the skin layer in one clean line parallel to the trachea, and then to gently separate the submandibular and parotid glands, also parallel to the trachea, to expose the muscle layer overlying the trachea. If voluntary movement is noted during the incisions, the procedure is halted until a deeper plane of anesthesia is achieved, and if necessary another 0.1 ml of urethane anesthetic will be administered i.p. The scissors are then used to separate the muscle layer parallel and directly over the trachea, thus revealing the trachea. Microscissors are then used to cut half-way through the trachea between the 4th to 5th tracheal cartilage, counting rings

from the larynx. A 20 g blunt tip luer cannula is inserted into the trachea, and then secured into place with 2-0 surgical silk ligature.

The tracheotomized animal is then connected to the ventilator via the canula luer, on a 37 degree C heated pad (the pad is heated by circulating water kept at 37 C). Mice are ventilated with 100% oxygen at 150 breaths per minute. The tail is taped to the heating pad to ensure the animal is kept in a straight line with the ventilator, but the 4 limbs are not restrained. Electrical leads are attached to three of the animal's limbs, which allow heart rate and waveform to be monitored by a window on the computer monitor. A rectal probe is then inserted to monitor body temperature. Monitoring the temperature, heart rate and waveform, along with the characteristics of the airway pressure tracing in a separate window, allows us to be sure that the animal is in a sufficient state of anesthesia after administration of the pancuronium bromide. Each mouse is then paralyzed with pancuronium bromide (0.8 mg/kg i.p., about 0.2 ml for a 20 gm mouse) to eliminate skeletal muscle movement which interferes with airway measurements.

Animals will be monitored during the procedure and should they show any signs of recovery from anesthesia, (e.g. eye and body movement) then the animal will be euthanized by overdose of sodium pentobarbital (> 200 mg/kg, i.p.).

The mouse will then be challenged with aerosols of MCh (up to 3 doses ranging from 12 to 50 mg/ml). This procedure has been titrated and used for over 15 years in Exemption 6 laboratory with no indication that the animals emerge from anesthesia or that the paralytic agent is reversed during the 30 minute treatment period. Surgical instruments and animal fur will be wiped down with 70% alcohol to keep contamination to a minimum. At completion of experiments the mice will be humanely euthanized while under urethane anesthesia by exsanguination and vital organ section (cutting of descending aorta and kidney). At conclusion of the procedure blood, BAL fluid, and lung tissue will be harvested.

The number of mice to be tested by the flexivent (Category D) includes all mice in this LAPR (160 mice).

No treatment-related deaths are expected and none have been encountered in over 15 years of experience in approved LAPRs treating mice with allergen and PM.

Multichamber Flexivent:

Scireq (recently acquired by emka Technologies) has developed a new 4-mouse multi-subject extension (MSX). This MSX system may also be used to assess pulmonary responses in anesthetized tracheotomized mice. Assessment of multiple subjects at one time will allow for more efficient assessments of pulmonary function. Time-of-day effects will be minimized with this system. Assessments using the MSX are similar to that of the single chamber system, except that monitoring of heart rate and temperature are not possible with the MSX during the experiment because the mice are placed in sealed body plethysmographs, and electrocardiogram (ECG) leads and temperature probes cannot be placed through the plethysmograph cylinder. In using the one-mouse system (since no cylinder is needed), monitoring of heart rate and temperature have always shown stable responses and mice remain sufficiently anesthetized. With the MSX, after achieving anesthesia, cannulated mice will be attached to the ventilator port, the cylinder is attached to the faceplate, the mouse is further monitored for required anesthesia, and breathing volume and pressure are monitored on the computer screen. If limb or body movements are observed, the plethysmograph cylinder can be removed and either further anesthesia administered (urethane, up to 2000 mg/kg, or the mouse will be removed from the system and euthanized by overdose of sodium pentobarbital (approx 200 mg/kg, i.p.). After ensuring proper anesthesia, the cylinder will be opened, neuromuscular blockade administered i.p. (pancuronium bromide, 0.8 mg/kg i.p.), and ECG leads will be attached to 3 limbs in a lead II configuration for 30 seconds to determine heart rate (beats/second). The leads will be removed, the cylinder closed and mice challenged with methacholine aerosol. At the end of the 25 minute assessment, each cylinder will be removed one at a time, and ECG leads attached again for 30 seconds to determine heart rate. The heart rates before and after the methacholine challenge assessment will be recorded and used to assess the efficacy of urethane as adequate anesthesia. Based on past experience, as stated above, we expect no increases in heart rate at the end of the assessment compared with heart rate determined before the assessment. If any such increases are found, the PI will consult immediately

with the veterinarian to determine a more effective anesthetic protocol.

b. Anesthetic regimen (Drugs, dosages, volume, route of administration and delivery schedule). The use of paralytic or neuromuscular blocking agents w/o anesthesia is prohibited:

Mice are given urethane (1 g/kg i.p.), carefully tested to determine lack of responses to toe pinch, tracheotomized, and put on the ventilator (diluted urethane will be made fresh each time from a stock not more than 1 year old from purchase date). Mice are then paralyzed with pancuronium bromide (0.8 mg/kg i.p.) to eliminate skeletal muscle movement which interferes with measurements.

The flexivent 5.1 software provides continuous monitoring of the EKG signal from the mouse. We will carefully monitor the heart rate to insure that each mouse has a steady heart rate following the administration of the urethane anesthesia. Once the baseline heart rate is established after anesthesia (expected to be about 8 beats per second or 480 beats per minute), we expect from our experience that the heart rate will typically remain constant or slightly decrease during the course of the experiment. If the animal was waking from the anesthesia, heart rate may increase to 9-10 beats per second (540-600 bpm). We have rarely if ever observed this condition, but if we did observe an increase (defined as 30 seconds or longer of a 15% increase in heart rate over baseline); we would administer more urethane (up to a total of 1.5-2.0 g/kg i.p.). The heart rate will be continually monitored visually (and the waveform recorded electronically throughout the protocol), and it will be recorded on the data sheet for each mouse 1 minute after ventilation starts (the baseline), and then after every dose of MCh (which occurs at approximate 5 minute intervals). In addition temperature will be monitored by rectal probe and recorded electronically throughout the procedure, and the temperature will also be recorded in the data sheet along with the heart rate at the intervals noted in the prior sentence. Electronic recordings of heart rate, waveform, and body temperature are available for viewing after the experiment is ended.

At completion of the final methacholine challenge concentration, a subset of animals will receive atropine sulphate via intraperitoneal injection (120 ug or approx. 6 mg/kg) in 0.2 mL of saline to reverse bronchoconstriction, and thus improve recovery of lung instillate during the subsequent necropsy and bronchoalveolar lavage procedure [see Larcombe AN et al Respir Physiol & Neurobiology 161 (2008) 223-229].

c. Postoperative care (thermal support, special feeding, responsible personnel, removal of sutures/staples, frequency and duration of monitoring including weekend and holiday care):

n/a

d. Post operative analgesics (drugs, dosage, and volume and route of administration, frequency):

n/a

e. Will any animal be subject to more than one surgical procedure over the course of its lifetime, either here at NHEERL or elsewhere?

○ Yes ● No

f. Identify any surgical procedures performed at other institutions or by vendors:

none

8. Humane interventions (for treatments/procedures in all categories).

a. What resultant effects, if any, do the investigators expect to see following procedures or treatment? Please include transitory as well as permanent effects. Examples might include lethargy, ataxia, salivation or tremors. Indicate the expected duration of these effects.

In the event of deleterious effects, the Attending Veterinarian will be immediately notified for guidance on subsequent steps including euthanasia. Animals will be isolated in a clean control atmosphere and observed for recovery trends, and may be transferred to the training colony if recovered. No deleterious effects of these non-surgical procedures, however, are expected.

b. State the criteria for determining temporary or permanent removal of animals from the study. Describe actions to be taken in the event of deleterious effects from procedures or chemical exposures. Describe actions to be taken in the event of clinical health problems not caused by procedures or exposures.

Any animals displaying signs of illness (weight loss of >10% occurs overnight, huddling, isolation with ruffled exterior, shivering, development of hindered movement, labored breathing and isolation etc) will be considered for permanent removal as per advice of the staff veterinarian.

9. Alternatives to pain and distress (Category D and E Procedures only). Provide narrative regarding the sources consulted to ascertain whether acceptable alternatives exist for potentially painful/distressful procedures. Include databases searched or other sources consulted, the date of the search and years covered by the search, and key words and/or search strategy used. Assistance with searches is available through the EPA Library Staff.

Searches of PubMed (covering 1960 to the present) were performed on 9/22/2015 for alternatives for pain and distress associated with this procedure. The following key words were used along with mouse pulmonary function (flexiVent): animal testing alternative, pain management, in vitro (model, technique), distress. No acceptable alternatives were found for these procedures.

SECTION C - Animal requirements

Describe the following animal requirements :

1. Indicate the number of animals required over the study period for this protocol. Please enter numbers only.

a. Animals to be purchased from a Vendor for this study:	160
b. Animals to be transferred from another LAPR:	0
LAPR Number that is the source of this transfer:	
c. Animals to be transferred from another source:	0
d. Offspring produced onsite (used for data collection and/or weaned):	0
e. TOTAL NUMBER of animals for duration of the LAPR	160

2. Species (limited to one per LAPR): Mouse/Mice

3. Strain: C57BL/6 mouse/mice and BALB/c

Describe special requirements for animals with altered physiological responses (e.g., genetically altered, aged)

Mice will be group housed for the majority of their stay in the facility. Cage changes will occur as usual, but may occur more frequently if needed.

4. Sources of animals:

May include Charles Rivers or Jackson Laboratories or other

5. Provide room numbers where various procedures will be performed on animals:

a). Mice will be housed in one of the animal housing rooms upon arrival Exemption 6 Exemption 6 or other available room) and during non-exposure periods.

b).During exposure, mice will be transferred in an original rack with mice housed in home cages to green floor inhalation exposure rooms (whole body or dual-chamber air or ozone exposures in – Exemption 6 Exemption 6 Once the exposures are complete, mice will be transferred to their home cages in the same rack and moved back to the animal holding room.

c). Immediately after the final day of exposure, using transfer cages with beta chips bedding and filtered cage tops, animals will be transferred to Exemption 6 Exemption 6 for Flexi-vent lung function testing, followed by necropsy.

6. Will any animals be housed in areas other than the animal facility longer than 12 hours? If so, state location. Such areas require prior IACUC approval as a satellite facility before LAPR can be reviewed.

No *Room Numbers:*

7. Describe any transportation and containment methods involved in moving animals between EPA buildings, or between EPA and other institutions (excluding any commercial shipments)

After the final exposure for the group, animals will be transferred from Bldg A 5th floor in containers with filtered cage tops to Bldg B 5th floor for "Flexi-Vent" testing and necropsy.

8. Describe any unusual housing or husbandry requirements, or acclimation requirements. Justify any treatment beginning less than 3 days after arrival.

n/a

9. Describe special assistance requested of the animal contract staff, including procedures and dosing. NOTE, this request must be submitted separately to the Animal Resources Program Office (ARPO)

none

10. Housing and Enrichment.

The IACUC encourages the use of environmental enrichment whenever possible (see IACUC website for details). Provide details on how the animals will be housed, including type of cage (e.g., solid bottom or wire screen), bedding material, number of animals per cage, and environmental enrichment. Note that housing rodents individually without environmental enrichment requires justification.

Animals will be group-housed (4 per cage) in solid bottomed-cages with beta chip bedding or other approved bedding. All mice will have access to enviro-dry with nestlets for enrichment purposes.

SECTION D - Agents Administered to Animals

1. Identify all hazardous and non-hazardous agents to be administered to living animals. For agents requiring a Health and Safety Research Protocol (HSRP), provide the title of the approved HSRP for each such agent. If no protocol is required for an agent deemed potentially hazardous (e.g. nanoparticles, recombinant DNA), describe the safety precautions to be used.
Provide maximum dosing levels and route-appropriate LD50s (where available) for each agent used for dosing.

1. Ozone inhalation exposures: Ozone will be generated and delivered at 0.8 ppm concentration to flow within the WBP or DCP devices. For comparison, the LC50 for ozone is 4.8 ppm in rats (4800 ppb/ 4 hours/ inhalation/ rat).

2. Urethane (maximum dosing = 2000 mg/kg; mouse oral LD50 = 2500 mg/kg; IARC group 2A carcinogen) is used for anesthetizing mice tested on the flexivent pulmonary function testing apparatus. The approved HSRP for this agents is #687 (Title: Allergenicity of Platinum Salts).

3. Alcohol – used externally only.

4. Pancuronium bromide (pharmaceutical grade; maximum dosing = 1 mg/kg; Mouse oral LD50 = 21 mg/kg due to respiratory skeletal muscle paralysis; paralysis is required to prevent interference with measurement of lung mechanics). Pancuronium bromide is reported to be a mutagen (equivocal results in Ames test) and has reproductive effects (fertility; post implantation mortality in rabbits).

5. Methacholine: (pharmaceutical grade; rat oral LD50 = 750 mg/kg). The mice will be challenged with increasing doses of methacholine via aerosol exposure (max) concentration 100 mg/ml in saline x 1 minute exposure). Prior experience has shown that these concentrations and duration of exposure are typically well-tolerated.

6. Atropine sulfate: (pharmaceutical grade) via intraperitoneal injection (120 ug or approx. 6 mg/kg) in 0.2 mL of saline to reverse bronchoconstriction. Oral LD50 in mice is 468 mg/kg.

7. Sodium pentobarbital (pharmaceutical grade; 50 mg/ml). Typical dosing for euthanasia will be 0.10 mL for a 20 gm mouse (or 195 mg/kg). The mouse oral LD50 is 137 mg/kg.

Researchers will handle all agents in accordance with good industrial hygiene and safety practices. PPE (such as gloves, lab coats, safety glasses, and masks) will be worn by personnel during experimental procedures in the animal facility and air pollutant exposure laboratories.

2. Describe compounds to be administered to animals.

a. Are all substances pharmaceutical grade? If not, provide a scientific justification for the use of non pharmaceutical grade compounds.

none

b. Describe any plans to administer human or animal tissues, blood or body fluids to the animals in the LAPR. Provide information to assure that such material is pathogen free. Indicate what safety precautions are necessary for handling the material.

n/a

c. Provide a statement regarding any safety precautions necessary for handling any of these materials.

n/a

NOTE: Any unresolved health/safety questions which arise during IACUC review of this LAPR will require consultation with the Safety, Health, and Environmental Management Office.

SECTION E - Personnel Training and Experience

1. Identify all project personnel conducting animal experimentation. Specify the techniques for which they have responsibility, and their relevant training and experience. Additional personnel may be added to the table below as a group (by Division) for Category C procedures. By so doing you are giving assurance that these personnel have received all required training and are qualified to perform the Category C techniques requested.

Use this area to type in additional personnel information not available in the table drop-down lists:

Hint: *The names in the first 2 lines of the table below are filled automatically from the Principal Investigator & Alternate Contact fields. A new line will be made available when a name is selected & upon leaving the name field (i.e. tabbing or clicking in another field).*

NAME	ROLE	SPECIFIC RESPONSIBILITY	RELEVANT TRAINING
Exemption 6	Principal Investigator	Plan study, prepare protocols, and oversee the experiment. Assist in animal handling, pulmonary function testing, and necropsy.	30 years as a Veterinarian, and 37 years of experience in laboratory animal research. All required NHEERL animal training courses are completed.
Exen Exemption 6 Exemption 6 Exemption 6	Post-Doc	Plan study, prepare protocols, and oversee the experiment. Assist in animal handling, pulmonary function	All required NHEERL animal training courses are completed. 3 years of experience working with lab animals.
Exemption 6	Associate Principal Investigator	Plan study, prepare protocols, and oversee the experiment. Assist	All required NHEERL animal training courses are completed. 30 years of experience working with lab animals.

		in animal handling, pulmonary function testing, and necropsy.	
Exemption 6	Post-Doc	Plan study, prepare protocols, and oversee the experiment. Assist in animal handling, testing, and necropsy.	All required NHEERL animal training courses are completed. 8 years of experience working with lab animals.
Exemption 6	Technical Staff	Assist in study planning, animal handling, ozone exposures, and pulmonary function assessments / data collection.	Twenty years of experience working with rats at NHEERL and other institu-tions, all required training completed.
Exemption 6	Technical Staff	Assist in study planning and necropsy.	All required NHEERL animal training courses are completed. 30+ years of experience working with lab animals.
Exemption 6	Technical Staff	Assist in study planning, animal handling, and necropsy.	All required NHEERL animal training courses are completed. 30 years of experience working with lab animals.
Exemption 6 Exemption 6 Exemption 6	Technical Staff	Assist in study planning, animal handling, and necropsy.	All required NHEERL animal training courses are completed. 20+ years of experience working with lab animals.
RTP-NHEERL	Tech Support	Category C Procedures	All NHEERL required training is complete.

SECTION F - Animal Breeding Colonies

This section pertains to the breeding of animals for maintenance of ongoing animal colonies. Do not include breeding that is part of experimentation and accountable under Section C.

Describe:

1. Estimated number of breeding pairs and liveborn per year n/a

2. Breeding protocols and recordkeeping n/a

3. Methods for monitoring genetic stability n/a

4. Disposition of all offspring and retired breeders that are not used in accordance with the procedures described in this LAPR n/a

SECTION G - Euthanasia

1. When will the animals be euthanized relative to experimental procedures?

Mice will be euthanized immediately after the “flexivent” anesthetized forced osillometry testing.

2. Describe the euthanasia techniques:

Method(s): Euthanasia plus exsanguination
Agent(s): Urethane
Dose (mg/kg): 2000 mg/kg urethane
Volume: Approx. 0.3 – 0.4 ml
Route: Intraperitoneal

Source(s) of information used to select the above agents/methods:

_ Veterinary Staff
IACUC, Personal Experience, Common Agents for Anesthesia & Euthanasia, literature as noted.

3. Provide justification and references for any euthanasia agent or method that is not consistent with recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (e.g., cervical dislocation or decapitation without anesthesia; cervical dislocation in rodents weighing more than 200 grams).
None

4. Describe how death is to be confirmed.
Vital organ section

SECTION H - Disposition of Used and Unused Animals

Describe the disposition of any animals remaining after project completion.
Transferred to another study or the training colony
The IACUC encourages investigators to reduce the overall number of animals used at NHEERL. Would you consider transferring any unused animals from this LAPR to another approved LAPR?
● Yes ○ No

SECTION I - Assurances

1. Animals will not be used in any manner beyond that described in this application without first obtaining formal approval of the IACUC.
2. All individuals involved in this project have access to this application, are aware of all EPA policies on animal care and use, and are appropriately trained and qualified to perform the techniques described.
3. Thorough consideration of the three "R"'s (Replacement, Reduction, Refinement) has been given, as applicable, to a. the use of animals, and b. procedures causing pain or distress (with or without analgesia/anesthesia), including death as an endpoint. The minimum number of animals required to obtain valid experimental results will be used.
4. The Attending Veterinarian has been consulted in regard to any planned experimentation involving pain or distress to animals.
5. The IACUC and Attending Veterinarian will be promptly notified of any unexpected study results that impact the animals' well-being, including morbidity, mortality and any occurrences of clinical symptoms which may cause pain or indicate distress.
6. All procedures involving hazardous agents will be conducted in accordance with practices approved by the Safety, Health, and Environmental Management Office.
7. I certify that I am familiar with and will comply with all pertinent institutional, state and federal rules and policies.
8. The IACUC has oversight responsibilities for animal care and use, and may request consultation or feedback regarding the conduct of in vivo procedures, progress and accomplishments, and any problems encountered.

EPA Principal Investigator	Certification Signature Date
Exemption 6 Exemption 6	09/02/2015

Submitted: 09/02/2015

Certification:

Certification by EPA Supervisor (Branch Chief or Division Director) that the project described herein has been reviewed and approved on the basis of scientific merit:

Branch Chief/Division Director	Approval Date	Phone Number	Division	Mail Drop
Exemption 6	09/03/2015 by Exemption 6/RTP/USEPA/US	Exemption 6 Lotus Notes Address Exemption 6/RTP/USEPA/US	EPHD Branch CIB	MD Submitted to Branch Chief for Approval 09/02/2015 06:58 PM

ATTACHMENTS

Table of Experimental Design.docx 18-09-004 LAPR PI Response.pdf 18-09-004 LAPR PI resp2.pdf

Actions

First Update notification sent: 07/27/2016
Second Update notification sent:
First 2nd Annual notification sent:
08/07/2017
Second 2nd Annual notification sent:

1st Expiration notification sent:
2nd Expiration notification sent:

History Log: